

# Differential response of fetal and adult fibroblasts to cytokines: cell migration and hyaluronan synthesis

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## SUMMARY

Previous studies have indicated that fetal skin fibroblasts display an elevated level of migratory activity compared to adult cells and that this may result from inherent differences in the production of hyaluronan (HA) by these cells. Data presented in this communication indicate that the elevated level of fetal fibroblast migration into 3D-collagen gels and HA synthesis by these cells were not affected by epidermal growth factor (EGF), platelet-derived growth factor (PDGF), acidic fibroblast growth factor (aFGF) or basic fibroblast growth factor (bFGF). In contrast, both cell migration and HA synthesis by fetal fibroblasts were inhibited by transforming growth factor-beta1 (TGF- $\beta$ 1). Adult fibroblasts responded to these cytokines in a distinct fashion: i.e. cell migration and HA synthesis were stimulated by EGF, PDGF, aFGF and bFGF, but remained unaffected by TGF- $\beta$ 1. Gel-filtration chromatography revealed that these effects of cytokines on HA synthesis were predominantly confined to the production of high molecular mass (>10<sup>6</sup> kDa) species. Co-exposure of cells to both cytokines and *Streptomyces* hyaluronidase revealed that (1) the elevated migration of control fetal fibroblasts was inhibited by hyaluronidase, (2) this inhibition was partially

restored by co-exposure to EGF, PDGF, aFGF and bFGF, but remained unaffected by TGF- $\beta$ 1, (3) the migration of control adult fibroblasts was unaffected by hyaluronidase and partially stimulated by EGF, aFGF and bFGF (when compared to the effects of these cytokines on cells cultured in the absence of hyaluronidase) and (4) neither PDGF nor TGF- $\beta$ 1 affected the migration of hyaluronidase-treated adult cells. Linear regression analysis revealed a significant correlation between cell migration and HA synthesis by both fetal and adult fibroblasts in the presence and absence of cytokines ( $r^2=0.9277$ ,  $P<0.0001$ ), with the exception of adult fibroblasts exposed to PDGF. Taken together, these findings suggest that (1) the migration of fetal and adult fibroblasts is differentially modulated by exogenous cytokines and (2) with the possible exception of the effects of PDGF on adult fibroblasts, cytokine-induced modulation of cell migration appears to utilise both HA-dependent and HA-independent pathways.

Key words: fibroblast, cell motility, cytokine, growth factor, hyaluronan, wound healing, cell-matrix interaction, human, EGF, TGF- $\beta$

## INTRODUCTION

Cell migration is a prominent feature of development. In contrast, tissue cells in the adult tend to lead a more sedentary existence, although (to various degrees) they remain capable of re-expressing a 'fetal-like' mode of elevated migratory activity during the course of pathological processes such as wound healing. In this regard, previous studies in various tissue culture assays of cell motility have confirmed that fetal fibroblasts display enhanced migratory activity compared to their adult counterparts (Schor et al., 1985; Chen et al., 1989; Kondo et al., 1993; Kondo and Yonezawa, 1995). The persistence of this behavioural difference in vitro suggests that it is not simply a response to differences in the fetal and adult tissue environment, but rather reflects a developmentally regulated alteration in intrinsic cell phenotype.

Hyaluronan (HA) is a glycosaminoglycan consisting of D-glucuronic acid (1- $\beta$ 3) N-acetyl-D-glucosamine (1- $\beta$ 4) disaccharide repeats (Laurent and Fraser, 1992). Various lines of evidence have implicated HA in the control of cell migration,

including (1) the relatively elevated presence of HA in fetal tissues containing actively migrating cell populations (Toole and Trelstad, 1971; Pratt et al., 1975; Bernanke and Markwald, 1979; Stern, 1984), (2) the effects of exogenous HA on cell migration in vitro (Bernanke and Markwald, 1979; Docherty et al., 1989; Ellis et al., 1992) and (3) the elevation of HA in adult tissues during pathological processes characterised by increased cell migration (Weigel et al., 1986, 1989; Burd et al., 1989; Toole et al., 1979; Turley and Tretiak, 1985; Dahl and Laurent, 1988; Longaker et al., 1991). The involvement of endogenously produced HA in determining the intrinsic difference in migratory behaviour between fetal and adult fibroblasts was suggested in a previous study (Chen et al., 1989); these authors demonstrated that fetal cells continued to synthesise higher levels of HA in vitro compared to adult cells and that this difference correlated with their enhanced migratory activity.

Cytokines also play a central role in the control of cell migration. In this regard, previous studies have demonstrated that such ubiquitous cytokines as epidermal growth factor

(EGF), platelet-derived growth factor (PDGF), transforming growth factor-beta (TGF- $\beta$ ) and fibroblast growth factor (FGF) stimulate cell migration in vitro (Seppä et al., 1982; Senior et al., 1986; Postlethwaite et al., 1987; Barrandon and Green, 1987; Buckley-Sturrock et al., 1989; Adelman-Grill et al., 1990; Stoker and Gherardi, 1991; Ellis et al., 1992; Kondo et al., 1993). Interestingly, the mechanisms mediating the effects of cytokines and matrix macromolecules on cell migration appear to be critically interdependent in the sense that (1) cytokines exert a direct effect upon the synthesis of matrix macromolecules, their cell surface receptors and matrix degrading enzymes, whilst (2) the macromolecular nature of the substratum exerts a modulatory effect upon cellular response to cytokines (Bade and Nitzgen, 1985; Nathan and Sporn, 1991; Kondo et al., 1992; Schor, 1994). With specific regard to the involvement of HA in the control of cell migration, we have previously reported that the effect of several cytokines on cell motility appears to be mediated by a primary modulation of HA synthesis (Schor et al., 1989; Ellis et al., 1992; Schor, 1994; Ellis and Schor, 1995).

In view of these various observations, the objectives of the present study have been (1) to compare fetal and adult fibroblasts with respect to the effect of various cytokines on both cell migration and HA synthesis and (2) to ascertain whether there is a correlation between these two parameters. Our results are discussed in terms of the potential significance of developmentally regulated differences in fibroblast response to cytokines in determining the distinct modes of fetal and adult wound healing.

## MATERIALS AND METHODS

### Reagents

Human platelet-derived TGF- $\beta$ , PDGF, aFGF and bFGF were purchased from R&D Systems, Ltd. (Cowley, Oxford, UK); EGF was purchased from Collaborative Research (Fred Baker Scientific, Runcorn, Cheshire, UK). D-[ $^3$ H] glucosamine hydrochloride (22 Ci/mmol) was obtained from Amersham International plc (Amersham, Bucks, UK). Hyaluronan (type 1, sodium salt), chondroitin-6-sulphate and hyaluronate lyase were obtained from Sigma Chemical Co. (Poole, Dorset, UK). Ultragold scintillation fluid was obtained from Canberra Packard (Pangbourne, Berkshire, UK). Eagle's Minimal Essential Medium (MEM), donor calf serum, sodium pyruvate, glutamine, non-essential amino acids, antibiotics and tissue culture plastic dishes were obtained from Gibco BioCult (Paisley, Scotland, UK). All other chemicals were obtained from BDH Chemicals (Poole, Dorset, UK) or Sigma Chemical Co. (Poole, Dorset, UK).

### Cell culture and preparation of collagen gels

The fetal and adult fibroblast lines used in this study were established in our laboratory by explant culture from skin biopsies (Ham, 1980). Stock cultures were maintained in Eagle's MEM supplemented with 15% (v/v) donor calf serum, 1 mM sodium pyruvate, 2 mM glutamine and non-essential amino acids at 37°C in a moist atmosphere containing 5% CO<sub>2</sub>. Cells were grown on 90 mm plastic tissue culture Petri dishes and passaged at a split ratio of 1:5 upon reaching confluence, 7-10 days after plating. All experiments were performed with fibroblasts between passage 10-18. Cultures used in this study were shown to be free of mycoplasma contamination by staining with Hoechst 33256.

Type I collagen was extracted from rat tail tendons in 3% acetic acid, dialysed for 2 days against distilled water, diluted to 2 mg/ml

and used to make 2 ml collagen gels in 35 mm plastic tissue culture dishes as previously described (Schor and Court, 1979).

### Migration assay

Collagen gels were overlaid with 1 ml of either SF-MEM (controls) or serum-free MEM containing 4 $\times$ the final concentration of cytokine. Confluent stock cultures were trypsinised, pelleted by centrifugation and resuspended in growth medium containing 20% donor calf serum at a concentration of 2.5 $\times$ 10<sup>5</sup> cells/ml; 1 ml samples of this inoculum were pipetted onto the cultures. Considering the 2 ml volume of the collagen gel, the 1 ml medium overlay and the 1 ml cell inoculum, this procedure gives a final concentration of 5% serum in both control and cytokine-containing cultures. The cultures were incubated for 4 days and the percentage of fibroblasts found within the 3D-gel matrix was then ascertained by microscopic observation of 15-20 randomly selected fields, as previously described (Schor, 1980). Replicate gels were counted for each experimental condition.

### Metabolic labelling and determination of total labelled HA

Glycosaminoglycans (GAGs) synthesised by the fibroblasts were metabolically labelled by incubating cultures with 2.5  $\mu$ Ci/ml of <sup>3</sup>H-glucosamine. These cultures were separated into medium and pericellular fractions, as previously described (Ellis et al., 1992). In this protocol, the medium and gel were transferred to a centrifuge tube and spun at 3000 rpm for 20 minutes at 4°C. The medium was removed and the compacted gel resuspended in 2 ml of phosphate-buffered-saline. Centrifugation was repeated and the supernatant combined with the previous one to form the medium fraction. The pericellular fraction was obtained by extracting the cell layer with 2 ml 50 mM Tris-HCl, 4 M guanidinium chloride, pH 7.4, for 24 hours at 4°C. The insoluble material was removed by centrifugation at 12000 g for 2 minutes in a microcentrifuge and the supernatant was collected as the pericellular fraction. The medium and pericellular fractions were dialysed against 0.1 M sodium acetate, 5 mM EDTA, pH 5.5 for 48 hours (with two changes of solution per day).

The dialysate volumes were measured and six 200  $\mu$ l samples were taken of each sample. 20  $\mu$ l of *Streptomyces* hyaluronidase (HA lyase: EC.4.2.2.1), at a final concentration 0.5 U/ml in acetate buffer, was added to three of these, whilst 20  $\mu$ l of acetate buffer was added to the remaining three controls. All samples were incubated for 17 hours at 37°C; after boiling for 1 minute to inactivate the enzyme, unlabelled carrier GAG was added (30  $\mu$ l 1% chondroitin sulphate/0.5% hyaluronate in PBS). The undigested GAGs were precipitated with four volumes of 1.3% potassium acetate in 100% ethanol at -20°C for 3 hours (Underhill and Toole, 1979). The precipitate was sedimented by centrifugation at 12000 g for 5 minutes, the supernatant removed and the pellet washed in 1 ml 75% ethanol and sedimented as above. The supernatant was again removed and the pellet redissolved in 100  $\mu$ l of 1 M sodium hydroxide and boiled for 3 minutes. The sodium hydroxide was neutralised with 100  $\mu$ l of 1 M hydrochloric acid and radioactivity determined using a Packard scintillation counter. The DPM associated with HA was taken as the difference between the counts in the control and enzyme-digested preparations.

We consistently found that less than 5% of the total synthesised HA was present in the pericellular fraction under all experimental conditions; data are consequently only presented for HA present in the medium fraction. We have previously confirmed the reliability of estimating HA synthesis by radiolabel incorporation by demonstrating the close correlation between the results obtained by this method and biochemical determination of chemical mass (Chen et al., 1989).

### Molecular size distribution of labelled HA

The molecular size distribution of labelled material was analysed by Sepharose CL-2B gel filtration chromatography of 200  $\mu$ l samples; columns had an internal diameter of 10 mm, contained 25 ml of gel and were equilibrated with 50 mM Tris-HCl, 4 M guanidinium chloride, pH 7.4. Samples of labelled medium and pericellular

fractions were first exhaustively dialysed against 50 mM sodium acetate, 10m M NaCl, pH5.5. Representative samples were digested prior to chromatography with 2 U of *Streptomyces* hyaluronidase for 17 hours in order to identify the radiolabelled HA. The column was eluted with Tris/guanidinium hydrochloride buffer at 6 ml/hour. Fractions (0.5 ml) were collected and radioactivity determined with a Packard scintillation counter.  $V_{DB}$  (void volume of column) and  $V_t$  (total column volume) were determined by the elution of dextran blue and sodium dichromate, respectively. The relative proportions of high (empirically defined as  $>2 \times 10^6$  kDa) and low molecular mass HA were estimated by cutting out and weighing the peaks in the radioactivity profile associated with the void (estimated by  $V_{DB}$ ) and included volumes, respectively.

### Statistical analysis

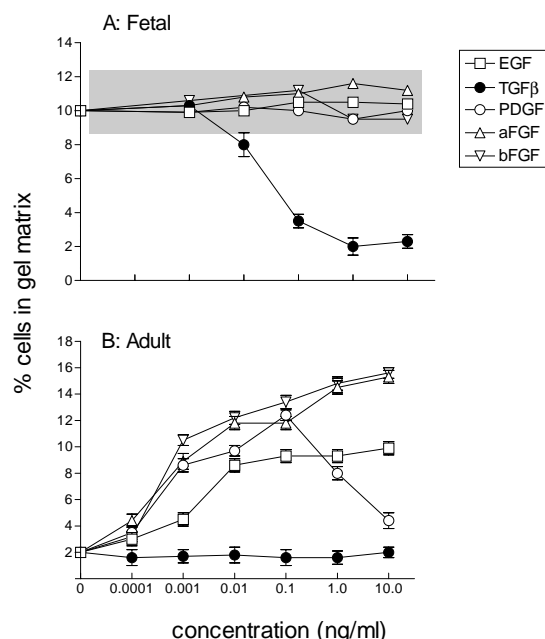
All statistical tests were performed using *Prism 2* graphics software (GraphPad Software, San Diego, California). The two-tailed *t*-test was employed.

## RESULTS

### Differential effect of cytokines on fetal and adult fibroblast migration

Fetal and adult fibroblasts were plated onto the surface of 3D-collagen gel substrata in the absence (control) and presence of the cytokines to be tested. The effects of cytokines on both cell migration and HA synthesis were determined as indicated in Materials and methods.

We have previously reported that confluent fetal fibroblasts migrate into 3D-collagen matrices to a significantly greater extent than adult cells (Chen et al., 1989). Data obtained with a representative fetal and adult fibroblast line (Fig. 1) confirm this observation and further reveal that these fibroblasts also differ with respect to cytokine modulation of cell migration. In this regard, EGF, PDGF, aFGF and bFGF (in the tested concentration range of 0.1 pg-10 ng/ml) did not affect the constitutively elevated migration of fetal fibroblasts; in contrast, all of these cytokines stimulated the relatively low migration of confluent adult fibroblasts. The effect of PDGF on adult fibroblast migration was unique in that it was the only cytokine to exhibit a bell-shaped dose-response curve in this concentration range. Fetal and adult fibroblasts also



**Fig. 1.** Dose response of a representative fetal and adult fibroblast line to cytokines. Representative fetal (F104) and adult (FSF44) fibroblast cell lines were plated at confluent cell density onto the surface of a 3D-collagen gel in the presence of various concentrations of the indicated cytokines. The number of cells that had migrated into the collagen matrix was determined on day 4. The shaded area indicates the range of error bars for results obtained with fetal fibroblasts in the presence of EGF, PDGF, aFGF and bFGF.

differed in terms of their response to TGF- $\beta$ ; this cytokine inhibited fetal fibroblast migration, but had no apparent effect on adult cells.

Data concerning the effect of a single concentration of cytokine on the migration of three fetal and three adult fibroblast lines are summarised in Table 1 (expressed as absolute levels of migration) and Fig. 2A (expressed as migration relative to control). These data indicate that fetal and adult fibroblasts consistently exhibit a differential migratory response to the examined cytokines.

**Table 1.** Effect of cytokines on migration of adult and fetal fibroblasts

Cell line	Control	Cytokine				
		EGF	TG $\beta$ 1	PDGF	aFGF	bFGF
<b>Fetal</b>						
FS6	15.5 $\pm$ 1.8	17.4 $\pm$ 0.9	3.9 $\pm$ 1.5	17.6 $\pm$ 1.1	17.1 $\pm$ 0.1	18.0 $\pm$ 0.9
F105	13.5 $\pm$ 2.9	14.4 $\pm$ 2.6	7.1 $\pm$ 2.0	17.6 $\pm$ 1.1	12.6 $\pm$ 1.0	12.0 $\pm$ 2.5
F104	13.7 $\pm$ 3.2	15.4 $\pm$ 1.2	4.4 $\pm$ 2.0	15.7 $\pm$ 3.3	10.9 $\pm$ 0.4	10.5 $\pm$ 0.2
Mean	<b>14.2<math>\pm</math>0.9</b>	<b>15.7<math>\pm</math>1.2</b>	<b>5.1<math>\pm</math>1.4</b>	<b>16.9<math>\pm</math>0.9</b>	<b>13.5<math>\pm</math>2.6</b>	<b>13.5<math>\pm</math>3.2</b>
<b>Adult</b>						
FSF37	2.3 $\pm$ 0.7	11.5 $\pm$ 1.7	2.4 $\pm$ 0.2	8.4 $\pm$ 1.7	12.6 $\pm$ 1.2	11.6 $\pm$ 0.4
FSF44	3.7 $\pm$ 1.1	10.8 $\pm$ 0.5	3.7 $\pm$ 0.1	9.1 $\pm$ 0.3	9.9 $\pm$ 0.7	10.4 $\pm$ 0.7
TSK319	2.5 $\pm$ 0.4	9.2 $\pm$ 0.5	2.3 $\pm$ 0.1	8.2 $\pm$ 0.3	9.0 $\pm$ 0.5	9.5 $\pm$ 0.5
Mean	<b>2.8<math>\pm</math>0.6</b>	<b>10.5<math>\pm</math>1.0</b>	<b>2.8<math>\pm</math>0.6</b>	<b>8.6<math>\pm</math>0.4</b>	<b>10.5<math>\pm</math>1.5</b>	<b>10.5<math>\pm</math>0.9</b>

Data are presented for several adult and fetal fibroblast lines in the presence of EGF, TGF- $\beta$ , aFGF and bFGF (all at 10 ng/ml) and PDGF (at 1 ng/ml). Cell migration was determined microscopically on day 4, as described in Materials and methods.

**Table 2. Effect of cytokines on HA synthesis by adult and fetal fibroblasts**

Cell line	Control	Cytokine				
		EGF	TG $\beta$ 1	PDGF	aFGF	bFGF
<b>Fetal</b>						
FS6	136.1 $\pm$ 6.9	144.7 $\pm$ 8.5	58.7 $\pm$ 6.1	137.4 $\pm$ 8.5	130.2 $\pm$ 1.6	150.6 $\pm$ 1.9
F104	98.8 $\pm$ 2.0	153.5 $\pm$ 17.7	32.3 $\pm$ 1.9	173.2 $\pm$ 0.3	133.4 $\pm$ 2.1	107.7 $\pm$ 0.8
F105	92.9 $\pm$ 12.6	134.6 $\pm$ 17.3	31.5 $\pm$ 6.1	130.6 $\pm$ 2.9	137.7 $\pm$ 0.6	158.9 $\pm$ 2.6
Mean	<b>109.3<math>\pm</math>19.3</b>	<b>144.3<math>\pm</math>7.7</b>	<b>40.8<math>\pm</math>12.6</b>	<b>147.1<math>\pm</math>18.7</b>	<b>133.8<math>\pm</math>3.1</b>	<b>139.1<math>\pm</math>22.4</b>
<b>Adult</b>						
FSF37	29.9 $\pm$ 2.5	64.6 $\pm$ 6.1	31.3 $\pm$ 2.6	140.3 $\pm$ 9.2	104.8 $\pm$ 9.6	107.6 $\pm$ 8.6
FSF44	49.2 $\pm$ 3.6	110.6 $\pm$ 2.6	44.7 $\pm$ 6.5	95.6 $\pm$ 5.3	125.3 $\pm$ 4.6	111.1 $\pm$ 2.9
SK319	30.7 $\pm$ 1.9	73.5 $\pm$ 7.5	29.5 $\pm$ 1.9	85.2 $\pm$ 1.6	74.1 $\pm$ 2.3	60.6 $\pm$ 2.1
Mean	<b>36.6<math>\pm</math>8.9</b>	<b>82.9<math>\pm</math>19.9</b>	<b>35.3<math>\pm</math>6.7</b>	<b>103.7<math>\pm</math>25.9</b>	<b>101.4<math>\pm</math>21.0</b>	<b>93.1<math>\pm</math>23.0</b>

Data are presented for three adult and three fetal fibroblast lines. Fibroblasts were plated at confluent cell density onto the surface of 3D-collagen gels in the presence of EGF, TGF- $\beta$ 1, aFGF and bFGF (all at 10 ng/ml) and PDGF (at 1 ng/ml). 2.5  $\mu$ Ci of  $^3$ H-glucosamine was present for the duration of the 4 day assay. Data are expressed as dpm $\times 10^{-3}/10^5$  cells.

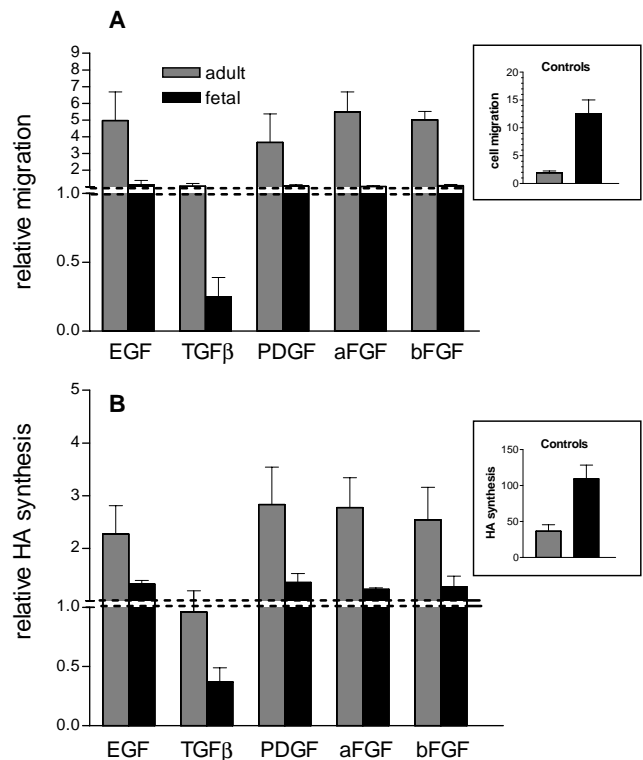
### Differential effect of cytokines on HA synthesis by fetal and adult fibroblasts

The effects of cytokines on HA synthesis by three fetal and three adult fibroblast lines are summarised in Table 2 (absolute levels of  $^3$ H-glucosamine incorporation) and Fig. 2B (relative level of incorporation). These data parallel those relating to cell migration and indicate that (1) confluent fetal fibroblasts synthesise more HA compared to adult cells, (2) EGF, PDGF, aFGF and bFGF had no significant effect on HA synthesis by fetal fibroblasts, but stimulated HA synthesis by adult cells and (3) TGF- $\beta$ 1 inhibited HA synthesis by fetal fibroblasts, but had no effect on adult cells.

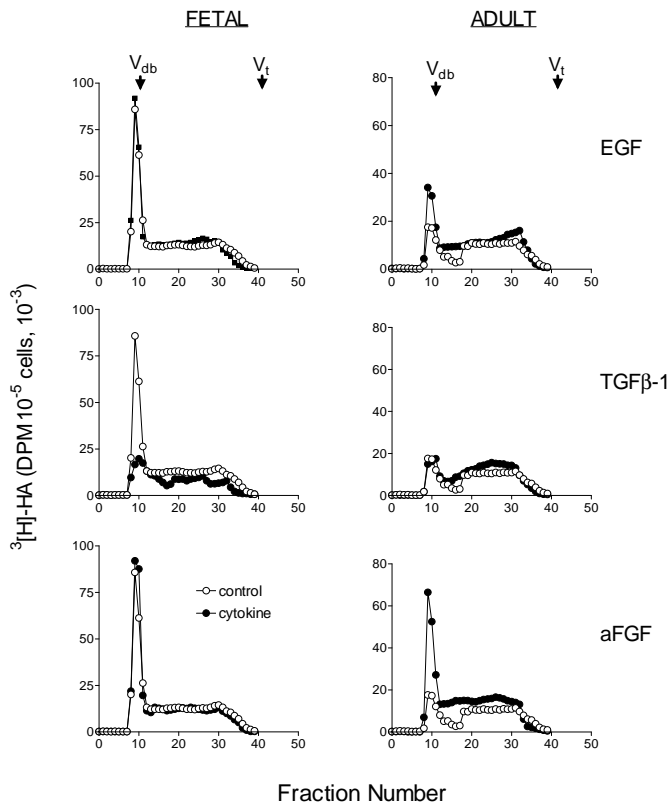
The effects of cytokines on the polydispersity of HA synthesised by fetal and adult fibroblasts are indicated in Fig. 3. These data indicate that EGF and aFGF had no effect on the relative synthesis of high and low molecular mass HA by fetal cells; identical results were obtained with PDGF and bFGF (data not shown). Adult fibroblasts responded to these cytokines in a distinct fashion in that EGF and aFGF stimulated the relative synthesis of high molecular mass HA; PDGF and bFGF behaved in an identical fashion (data not shown). TGF- $\beta$ 1 was again different from the other cytokines examined in that it inhibited the relative synthesis of high molecular mass HA by fetal fibroblasts, but did not affect high molecular mass HA synthesis by adult cells.

### Functional relationship between cell migration and HA synthesis

We have previously reported that the migration of confluent fetal fibroblasts is inhibited by *Streptomyces* hyaluronidase whilst the migration of adult fibroblasts remains unaffected (Schor et al., 1989). Data are presented in Fig. 4 concerning the effect of different concentrations of hyaluronidase on the migration of fetal and adult fibroblasts, either in the absence of cytokine (control) or co-exposed to aFGF. These observations confirm the differential sensitivity of control fetal and adult fibroblasts to hyaluronidase, and further indicate that (1) a plateau level of maximal inhibition of control fetal fibroblast migration was achieved between 0.1 and 1.0 Units/ml of hyaluronidase, (2) co-exposure of fetal fibroblasts to hyaluronidase (various concentrations) and aFGF (10 ng/ml) resulted in only a partial inhibition of migration, with a plateau level again achieved between



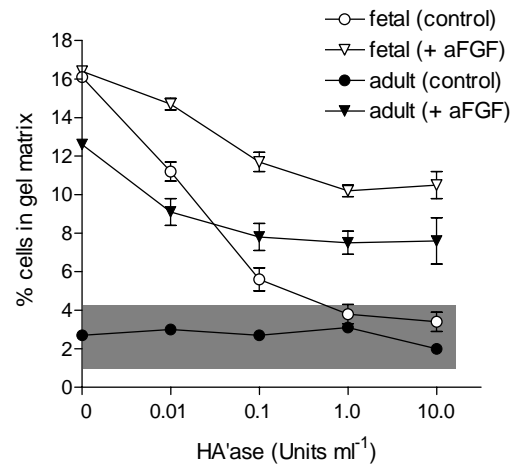
**Fig. 2.** Effect of cytokines on migration and HA synthesis of fetal and adult fibroblast lines. Three fetal and three adult fibroblast lines were plated at confluent cell density onto the surface of 3D-collagen gels in the absence (control) and presence of the indicated cytokines (at concentrations of 1 ng/ml for PDGF and 10 ng/ml for all others).  $^3$ H-glucosamine was added to all cultures. The percentage of cells present within the gel matrix and the amount of radiolabelled glucosamine incorporated in HA was measured after 4 days incubation, as described in Materials and methods. Data are expressed as migration relative to control (A) and HA synthesis relative to control (B). Data presented in the two insets compare control fetal and adult cells in terms of their absolute level of cell migration (percentage of cells within the gel matrix) and HA synthesis (d.p.m. $\times 10^{-3}$   $^3$ H-glucosamine/ $10^5$  cells), respectively.



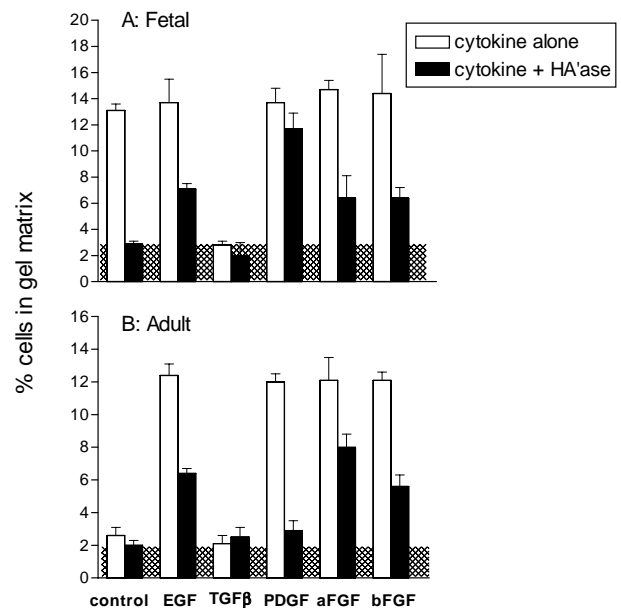
**Fig. 3.** Effect of cytokines on the size class distribution of HA synthesised by fetal and adult fibroblast lines. The polydispersity of HA synthesised by fetal and adult fibroblasts plated on the surface of a 3D-collagen gel was analysed using Sepharose CL-2B column chromatography, as described in the Materials and methods.  $V_{db}$  indicates the point at which dextran blue was eluted from the column and is an estimate of the void volume;  $V_t$  indicates the total column volume.

0.1 and 1.0 Units/ml hyaluronidase, (3) the migration of control adult fibroblasts was unaffected by any of the concentrations of hyaluronidase examined and (4) the migration of adult fibroblasts co-exposed to aFGF and hyaluronidase was partially inhibited, with a plateau level of maximal inhibition achieved between 0.01 and 0.1 Units/ml. Comparable data obtained with all the cytokines co-exposed to the maximal dose of hyaluronidase (10 Units/ml) are summarised in Fig. 5. These results indicate that (1) co-exposure of fetal fibroblasts to hyaluronidase and either EGF, aFGF or bFGF resulted in only a partial inhibition of cell migration, (2) hyaluronidase did not appear to inhibit the stimulatory effect of PDGF on fetal fibroblast migration and (3) co-exposure of adult fibroblasts to hyaluronidase and cytokines resulted in a partial stimulation of cell migration (relative to control) for all except PDGF. TGF- $\beta$ 1 had no effect on the migration of either fetal or adult fibroblast co-exposed to hyaluronidase.

Taken together, these data suggest that the effects of cytokines on the migration of fetal and adult fibroblasts are functionally related to effects of these cytokines on HA synthesis. Linear regression analysis (Fig. 6) confirms the predicted correlation between cell migration and HA synthesis ( $r^2=0.9277$ ;  $P<0.0001$ ). Note that the only outlying point represents data obtained with PDGF on adult fibroblasts.



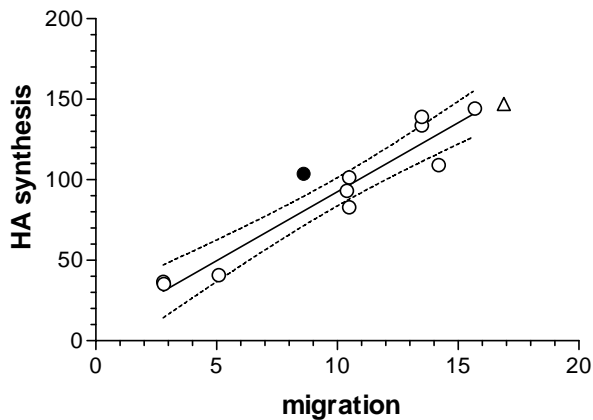
**Fig. 4.** The dose-response of control and aFGF-treated fetal and adult cells to *Streptomyces* hyaluronidase (hyaluronate lyase). Fetal and adult fibroblast lines were incubated with various concentrations of hyaluronidase in the absence (control) and presence of aFGF. Data are expressed as the percentage of cells present within the 3D-collagen gel matrix after a 4-day incubation period. The shaded area represents the spread of error bars for control adult fibroblasts.



**Fig. 5.** The effect of *Streptomyces* hyaluronidase (hyaluronate lyase) on the migration of fetal and adult fibroblasts and their response to cytokines. Fetal (A) and adult (B) fibroblast lines were plated onto the surface of 3D-collagen gels in the presence and absence of 10 Units/ml hyaluronidase and added cytokine (10 ng/ml EGF, TGF- $\beta$ 1, aFGF or bFGF, and 1 ng/ml PDGF). The number of cells that had migrated into the collagen gel were determined on day 4. The shaded area indicates migration achieved by control cells in the presence of hyaluronidase.

**DISCUSSION**

Data presented in this communication indicate that: (1) fetal and adult skin fibroblasts exhibit a differential response to all of the cytokines examined in this study (EGF, PDGF, TGF- $\beta$ 1,



**Fig. 6.** Correlation between HA synthesis and migratory activity of fetal and adult fibroblasts. The correlation between HA synthesis and cell migration of three fetal and three adult lines in the presence of cytokines were analysed by linear regression analysis ( $r^2=0.9277$ ;  $P<0.0001$ ). Open triangle, PDGF with fetal cells; filled circle, PDGF with adult cells; open circles, all remaining control and cytokine-treated cells.

aFGF and bFGF) in terms of both cell migration and HA synthesis, (2) cytokine-induced changes in the relative synthesis of high molecular mass HA account for the observed effects on total HA production (either stimulation or inhibition), (3) EGF, PDGF, aFGF and bFGF induce a partial stimulation of cell migration in both fetal and adult fibroblasts coincubated with *Streptomyces* hyaluronidase (with the notable exception of PDGF-treated adult fibroblasts) and (4) there is a significant correlation between HA synthesis and migration in both fetal and adult fibroblasts.

Cell migration has previously been reported to be affected by a wide spectrum of cytokines (Stoker and Gherardi, 1991; Schor, 1994). Many cytokines have also been shown to modulate the synthesis of HA (Bachem et al., 1989; Heldin et al., 1989; Toole et al., 1989; Postlethwaite et al., 1989; Ellis et al., 1992; Ellis and Schor, 1995; Papakonstantinou et al., 1995), as well as other species of matrix macromolecules. Direct comparison of fetal and adult fibroblasts in terms of their response to cytokines, with respect to these two parameters, has been limited. In this regard, we have reported that fetal fibroblasts produce a migration stimulating factor (MSF), which is not made by their normal adult counterparts, and that the migration of adult (but not fetal) fibroblasts into 3D-collagen gels is stimulated by the addition of exogenous MSF (Schor et al., 1988). Kondo and coworkers have reported similar differences between adult and fetal fibroblasts when migration was assessed by monitoring cell movement into a denuded scrape in a confluent cell monolayer; these authors found that the relatively elevated migration displayed by fetal fibroblasts in this assay resulted from their endogenous production of bFGF, whilst the migration of adult skin fibroblasts was preferentially stimulated by exogenous PDGF (Kondo et al., 1992, 1993).

The results presented in Fig. 3 indicate that the examined cytokines preferentially affect the synthesis of high molecular mass HA. This specificity of action may be of biological significance, as many of the effects of HA on target cells are dependent upon its size: these include the modulation of phagocytosis (Forrester and Balazs, 1980; Hankansson et al.,

1980; Hankansson and Vegne, 1985), angiogenesis (Feinberg and Beebe, 1983; West et al., 1985) and chondrogenesis (Kujawa et al., 1986).

The effects of *Streptomyces* hyaluronidase on the migration of control and cytokine-treated fibroblasts (Figs 4 and 5) are of particular interest. These data indicate that (1) degradation of endogenously produced HA results in a complete reduction of the constitutively elevated migration of control fetal fibroblasts to that characteristic of adult cells, (2) the four 'stimulating cytokines' (i.e. EGF, PDGF, aFGF and bFGF) produced a partial restoration of fetal fibroblast migration in the presence of hyaluronidase and (3) with the exception of PDGF, these same cytokines also produced an approximate 50% maximal stimulation of adult fibroblasts co-exposed to hyaluronidase. These observations are consistent with the interpretation that the 'stimulating' cytokines affect cell migration by both HA-dependent and HA-independent mechanisms, with the exception of PDGF and adult cells, where cytokine stimulation of cell migration appears to be totally HA-dependent.

The effects of HA on cell migration are mediated by interaction with its receptors, CD-44 and RHAMM (Thomas et al., 1992; Samuel et al., 1993). CD44 is a complex family of related molecules produced by alternative splicing (Ruiz et al., 1995). Individual CD44 isoforms display developmentally regulated differences in both cell expression and ligand affinity. Signal transduction elicited by ligation of RHAMM involves pp60<sup>c-src</sup> tyrosine phosphorylation of the focal adhesion kinase pp125<sup>FAK</sup> (Hall et al., 1994; Hall and Turley, 1995). Ongoing studies are concerned with definitively identifying the receptor(s) involved in mediating the effects of both cell-produced and exogenous HA on fibroblast migration into 3D-collagen matrices and the signal transduction cascades elicited by their ligation.

TGF- $\beta$  did not stimulate migration or HA synthesis by either fetal or adult fibroblasts. We have previously reported that the effect of TGF- $\beta$  on adult cell migration and HA synthesis is substratum-dependent (Ellis and Schor, 1995), i.e. it stimulates cell migration and HA synthesis by cells cultured on a conventional plastic substratum, but (as indicated in this communication) does not affect adult cells cultured on native collagen gels. This substratum-dependence appears to be a common feature of cytokine action (Schor, 1994).

Fetal and adult fibroblasts differ with respect to a number of other fundamental aspects of cell behaviour, including the production of various cytokines (Clemmons, 1983; Stoker et al., 1987; Schor et al., 1988; Grey et al., 1989; Kondo et al., 1993; Kondo and Yonezawa, 1995; Locci et al., 1993), the synthesis of particular isoforms of matrix macromolecules (Matsura and Hakomori, 1985; French-Constant et al., 1989), the presence of fetal specific antigenic determinants (Azzarone et al., 1984), the activity of matrix synthesising enzymes (Duncan et al., 1992) and the secretion of matrix degrading enzymes (Sottile et al., 1989; Basset et al., 1990). Taken in conjunction with the distinct effects of cytokines on cell migration and HA synthesis described in this communication, such differences may be of relevance to a number of significant biological differences between fetal tissues and their adult counterparts. For example, adult wound healing is essentially a 'reparative' process, commonly accompanied by scar formation (McPherson and Piez, 1988); in contrast, dermal wound healing in the early gestation fetus is regenerative in nature, with the resultant neo-



tissue being free from visible scarring (Adzick and Lorenz, 1994). Various data suggest that the regenerative nature of fetal wound healing results from the expression of particular phenotypic characteristics by fetal fibroblasts that are not shared by their adult counterparts (Lorenz et al., 1992; Schor et al., 1996). The important role played by HA in determining the scarless nature of fetal wound healing has been particularly well documented. In this regard, fetal wound healing matrix is characterised by persistent and higher levels of HA at the wound site compared to adult granulation tissue (Longaker et al., 1991). HA has also been reported to inhibit scar formation in healing adult wounds when applied topically (Hellström and Laurent, 1987) and reduce the packing density of collagen bundles (Scott and Hughes, 1986). Conversely, degradation of HA in healing fetal wounds was reported to result in scarring (Mast et al., 1992). These effects of HA on wound healing may be related to its reported stimulation of fibroblast migration (Ellis et al., 1992; Docherty et al., 1989), inhibition of fibroblast-induced collagen gel contraction (Hauglee et al., 1994) and modulation of collagen synthesis (Mast et al., 1993). The intrinsic differences between adult and fetal fibroblasts with respect to the modulation of HA synthesis (and cell migration) may be relevant in this context.

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